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Sugar acts as a regulatory signal on the wound-inducible expression of *SbHRGP3*::*GUS* in transgenic plants

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Abstract *SbHRGP3* encodes an HRGP whose expression is correlated with the cessation of root elongation in soybean. The wound-inducible expression of *SbHRGP3* interestingly requires sucrose although wounding alone induces the expression of many *HRGP* genes. To examine whether sugar serves as a specific signal on the wound-inducible expression or whether sugar is required to provide ATP, we examined *SbHRGP3*::*GUS* expression in transgenic tobacco plants. Various oligosaccharides including non-metabolizable sugar induced *SbHRGP3*::*GUS* expression in transgenic plants. The inhibitors of photosynthesis and of cellular respiration did not affect the wound-inducible expression of *SbHRGP3*::*GUS*. However, the induction was significantly affected by PCMBS, an inhibitor of active apoplastic phloem loading of sucrose, suggesting that *SbHRGP3*::*GUS* expression in phloem tissues requires translocated sucrose. We therefore propose that sugar acts as a specific regulatory signal on the wound-inducible expression of *SbHRGP3*, rather than acting as a simple provider of ATP.

Keywords Cell wall protein · Sugar · Signal inducer · Wound-induction · Extensin

Abbreviations *ATP:* Adenosine triphosphate · *DCMU:* 3-(3,4-Dichlorophenyl)-1,1-dimethylurea · *DTT:* Dithiothreitol · *HRGP:* Hydroxyproline-rich glycoprotein · *GUS:* β-Glucuronidase · *MU:* 4-Methylumbelliferone · *MUG:* 4-Methylumbelliferyl ß-glucuronide · *PCMBS: p*-Chloromercuribenzenesulphonic acid

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Introduction

Extensins are a family of HRGPs and constitute the major protein components in cell walls of dicot plants (Showalter 1993). The plant proteins not only contribute to the structural support of the cell wall but also to plant defence, helping to protect against pathogen attack or mechanical wounding (Wycoff et al. 1995). In spite of their structural role, expression of the extensin genes is not constitutive, but rather regulated in a tissue-specific (Wu et al. 2001) and development-specific manner (Hall and Cannon 2002). Furthermore, expression of the genes is affected by many cellular and environmental factors, which include mechanical wounding, fungal infection, viral infection, elicitors, ethylene treatment, and developmental signals (Memelink et al. 1993; Merkouropoulos et al. 1999; Parmentier et al. 1995; Wycoff et al. 1995).

Localization and accumulation of several cell wall proteins have been characterized in soybean (Ahn et al. 1996; Hong et al. 1994; Ye and Varner 1991). Expression of extensin genes is generally observed in stems and roots in healthy plants, being especially specific to phloem and cambium tissues (Showalter 1993). Other *HRGP* genes have been highly expressed in cambium cells of stems and their expression has also been observed in petioles, seed coats, and young hypocotyls (Ye et al. 1991; Ye and Varner 1991). In contrast, soybean proline-rich proteins and glycine-rich proteins, other major components of the cell wall, are localized in a limited number of cell types of a particular organ (Ringli et al. 2001; Suzuki et al. 1993). These data suggest that the cell wall proteins are different in each cell type and their expression is differentially regulated in soybean.

SbHRGP3 is a root-specific extensin gene with two distinct repeat motifs that correspond to P3- and P2-type

motifs (Ahn et al. 1996). *SbHRGP3* is a member of a new group of the extensin gene family with two distinct domains in a single polypeptide. Its expression is closely correlated with soybean root development, especially in the maturation stage (Ahn et al. 1998). Expression of *SbHRGP3* is upregulated in the region where the soybean root is not further elongated, which suggests that it is involved in cessation of root elongation in the maturation zone. *SbHRGP3* was wound-inducible as observed in many other extensin genes; however, its expression required sucrose, which is the first reported case in the extensin gene family. This result raises the possibility that sucrose may act as an inducing signal on *SbHRGP3* expression.

Sugars including sucrose affect various events during cell differentiation and growth in plants. For example, sugars negatively regulate hypocotyl elongation in *Arabidopsis* (Jang et al. 1997). Genes essential for the biosynthesis of ethylene are activated by sugars (Gibson et al. 2001), whereas glucose treatment destabilizes the alpha-amylase mRNA (Loreti et al. 2000). Sucrose also represses plastocyanin gene expression and the far-red light-induced cotyledon opening, suggesting a close interaction between sucrose and light signalling pathways (Dijkwel et al. 1997).

Sugar levels in plants vary widely depending on the tissues and developmental stages. However, it appears that the sugar levels are tightly regulated in plants (Jang and Sheen 1994). For instance, high sugar levels repress the expression of the genes for sugar production and induce the expression of the genes for storage and utilization. In contrast, sugar depletion exerts the opposite effects. The repression of expression of the genes involved in photosynthesis is one of the best-characterized examples (Koch et al. 2000; Van Oosten et al. 1997). Although sugars obviously affect growth and development in plants (Sheen et al. 1999; Wobus and Weber 1999), it is not clear whether sugars act as a specific regulatory signal or whether they merely disturb cellular metabolism and cause osmotic stress. Since sugar levels do not always correlate with sugar-regulated gene expression, therefore, it has also been suggested that metabolic flux, rather than accumulated sugar levels, is important in triggering sugar-responsive gene expression (Borisjuk et al. 2003).

In this study, we investigated the effect of sugars on the wound-inducible expression of *SbHRGP3*, an extensin gene involved in the maturation of soybean roots. To assess the role of sugars, we tested various inhibitors blocking external and internal energy sources, and checked *GUS* expression driven by the *SbHRGP3* promoter in transgenic plants. The results showed that blocking of the energy metabolism did not affect the wound-inducible expression of *SbHRGP3*::*GUS*; however, the inhibition of apoplastic sucrose loading toward phloem significantly reduced induction of *SbHRGP3*::*GUS* expression. Furthermore, non-metabolizable sugars including mannose also induced its expression. These results suggest that although sugar is an important energy source and a structural component in plants, it primarily acts as a specific regulatory signal on the wound-inducible expression of *SbHRGP3* in transgenic plants.

Materials and methods

Plant materials

Soybean (*Glycine max* cv. Paldal) and tobacco (*Nicotiana tabacum* cv. Xanthi) were used for all experiments. The plants were grown at 28°C under long-day conditions [16/8-h (day/night) photoperiod]. The light was provided by daylight fluorescent lamps (100 μmol s^{-1} m^{-2}).

For RNA extraction from soybean seedlings, soybean seeds were germinated on wet filter paper and their seedlings were grown in a modified half-strength Hoagland solution under a 16/8-h (day/night) photoperiod at 28°C. The tissues harvested from 13-day-old soybean seedlings were used to isolate total RNA for Northern blot analyses.

SbHRGP3::GUS chimeric gene construction and plant transformation

The 5′ flanking region (0.9 kb) of *SbHRGP3* was amplified by polymerase chain reaction by using the EXTP2 primer (5′-GTGTGCATAAGCTTTCCACATGTCAC-3′) and the EXTP3 primer (5′-TACGGATCCTAGGAGAGGTAGTGTTGCCC-3′) that contained the underlined synthetic restriction sites. The amplified product was fused to a DNA fragment containing the *GUS* reporter gene and the nopaline synthase terminator. The recombinant plasmid was cloned into pGA482, a binary vector for plant transformation (An et al. 1988). The recombinant plasmid was introduced into tobacco plants via *Agrobacterium*-mediated leaf disc transformation (Horsch et al. 1986). Transformed shoots were selected on a Murashige and Skoog basal medium (Gibco BRL) supplemented with 200 mg l^{-1} kanamycin and 500 mg l^{-1} carbenicillin. Transgenic seeds in the R_1 generation were aseptically germinated on wet filter paper in Petri dishes under long-day conditions and were used for further analyses.

Induction of SbHRGP3::GUS expression

Leaves of transgenic plants were surface-sterilized with commercial bleach solution and were wounded with a razor blade. The leaf slices were floated on liquid Murashige and Skoog basal media with or without 3% sucrose for 24 h.

To examine the effect of wounding and sucrose on the expression of *SbHRGP3* in soybean roots, roots from 9-day-old seedlings were wounded with a razor blade and floated on a MS medium supplemented with 3% sucrose for 24 h. Total RNA was also prepared from wounded roots without sucrose and unwounded roots cultured in 3% sucrose. Unwounded roots cultured without sucrose were used as a negative control. The same strategies were applied to soybean leaves grown under field conditions to isolate total RNA.

To investigate the effect of other sugars on the wound-inducible expression of *SbHRGP3*, various sugars including non-reducing disaccharides (raffinose and sucrose), reducing disaccharide (maltose), and monosaccharides (mannitol, fructose, glucose, mannose, arabinose, ribose, and xylose) were tested. Wounded leaves of transgenic plants were floated in a medium supplemented with the sugars of an equal molar concentration to 3% sucrose. GUS activity was measured as described above.

To test external stimuli related to pathogen response, intact soybean leaves were soaked in 10 m*M* DTT, 1 m*M* salicylic acid, or 1 m*M* phosphate solution for 24 h and a Northern hybridization

Table 1 The inhibitors used in this study

Inhibitor	Mode of action	Inhibition	Reference
DCMU	Binding two sites in photosystem II and inhibiting electron donation	Photosynthesis	(Hsu et al. 1986)
PCMBS	Inhibition of active apoplastic phloem loading of sucrose		(Bush 1993)
Iodoacetate	Inhibition of 3-phosphoglyceraldehyde dehydrogenase	Glycolysis	(Brumback 1980; Kane and Buckley 1977)
Malonate	Inhibition of succinate dehydrogenase	Krebs cycle	(Igamberdiev et al. 1995)
Azide	Inhibition of mitochondrial electron transport and eventually inhibits the ATP production	Oxidative phosphorylation	(Vasilyeva et al. 1982)

analysis was performed to examine *SbHRGP3* mRNA accumulation.

Inhibition of cellular respiration and apoplastic sucrose transport

The inhibitors used in this study are summarized in Table 1; 20 m*M* DCMU was used to inhibit photosynthesis, which acts as an external energy source; 1 m*M* iodoacetate, 10 m*M* malonate, and 1 m*M* azide were used to inhibit cellular respiration including glycolysis, the Krebs cycle, and oxidative phosphorylation, respectively (Kane and Buckley 1977). Apoplastic sucrose transport toward phloem was blocked by treating leaves with 100 μM PCMBS. After the leaves of transgenic plants were wounded and floated on a medium supplemented with sucrose and one of these inhibitors for 24 h, spectrofluorometric assays were performed with the tissues.

Spectrofluorometric assay of GUS activity

Transgenic plants carrying *SbHRGP3*::*GUS* were treated as described above and floated on media for 24 h. Unwounded leaves floated on a medium with or without 3% sucrose and wounded leaves floated on a medium without sucrose were used as a control.

The leaves were then homogenized in extraction buffer [50 m*M* sodium phosphate, pH 7.0, 5 m*M* DTT, 1 m*M* EDTA, 0.1% (w/v) sarcosyl, 0.1% Triton X-100]. Thirty micrograms of protein from each sample was assayed in a spectrofluorometer; 1 m*M* MUG was used as a fluorogenic substrate. Excitation was performed at 365 nm and emissions were detected at 455 nm. Freshly prepared 100 n*M* MU and 1 μM MU were used as internal standards. After three independent experiments, mean values and their SDs were calculated.

Results

SbHRGP3::*GUS* expression reaches a maximal level at 24 h after the wound-induction

Because sucrose is required for the wound-induction of *SbHRGP3* (Ahn et al. 1996), the time course of induction of *SbHRGP3*::*GUS* expression was examined in transgenic plants. Leaves of transgenic plants carrying *SbHRGP3*::*GUS* were wounded with a razor blade and were floated on 3% sucrose solution. The leaf slices were harvested at various time points and their GUS activities were determined by the spectrofluorometric GUS assay. Wounded leaves of transgenic plants that were floated on a medium without sucrose were used as a negative control.

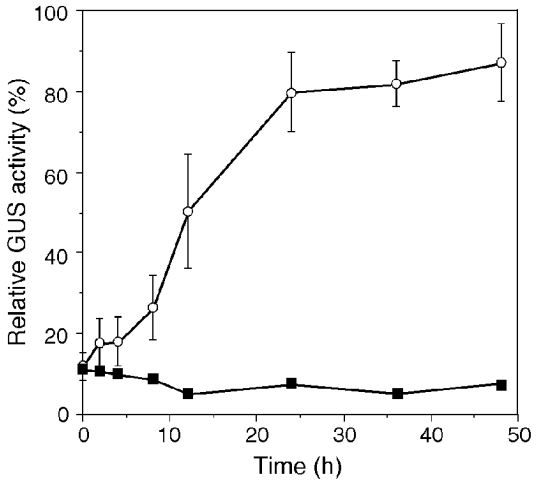

Fig. 1 Time course of *SbHRGP3*::*GUS* expression in transgenic plants. Leaves of transgenic plants were wounded and floated on a medium supplemented with 3% sucrose. After the leaves were harvested at various time points, their GUS activity was determined by the spectrofluorometric assay. Data were obtained from three independent experiments. *Bars* indicate SDs. ○ wounded transgenic plants floated on a medium supplemented with 3% sucrose, ■ wounded transgenic plants floated on a medium without sucrose

The spectrofluorometric assay showed that GUS activity increased after wounding and reached a maximal level at 24 h, which is an approximately sevenfold increase from the basal level (Fig. 1). This enhanced GUS activity remained until 48 h, showing that the induced expression of *SbHRGP3*::*GUS* was stably maintained. In contrast, the wounded leaves of transgenic plants without sucrose did not show any increased GUS activity until 48 h, which agrees with previous results (Ahn et al. 1996, 1998).

SbHRGP3::*GUS* expression was also induced by various sugars

Because the wound-inducible expression of *SbHRGP3* required sucrose, the effects of other sugars on the wound-inducible expression of *SbHRGP3* were examined in transgenic plants. Leaves of transgenic plants were wounded as described previously and floated on media

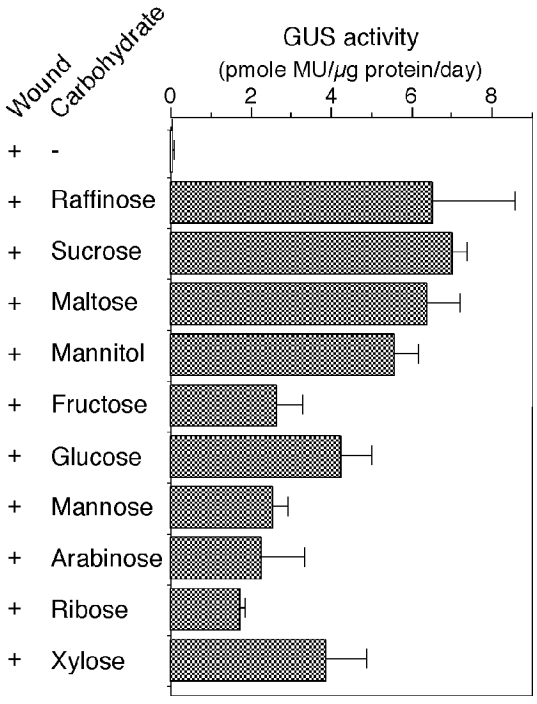

Fig. 2 Effects of various oligosaccharides on the wound-inducible expression of *SbHRGP3*::*GUS* in transgenic plants. Leaves of transgenic plants were wounded and floated on a medium supplemented with various oligosaccharides of a molar concentration equivalent to 3% sucrose solution. Data were obtained from three independent spectrofluorometric measurements. *Bars* indicate SDs of the experiments

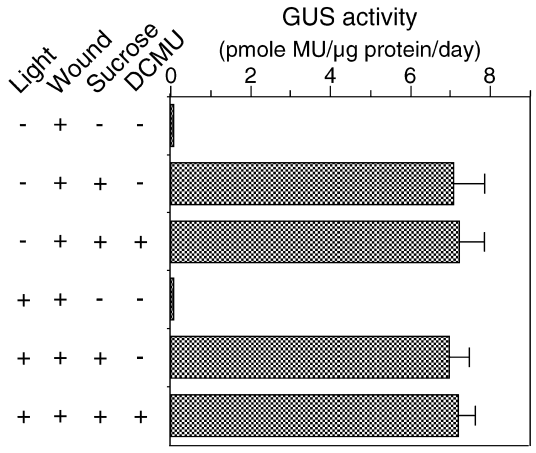

Fig. 3 Effects of DCMU, an inhibitor of the light reaction in photosynthesis, on *SbHRGP3*::*GUS* expression in transgenic plants. Treated (+) and untreated (−) leaf slices of transgenic plants were incubated for 24 h in a medium with or without the inhibitor. Thirty micrograms of protein extracted from each sample was assayed for GUS activity. Each mean value was obtained after three independent experiments. *Bars* indicate SDs

supplemented with various oligosaccharides of equivalent molar concentrations to 3% sucrose solution for 24 h.

The result showed that all the sugars used in this study induced *SbHRGP3*::*GUS* expression to variable degrees (Fig. 2). Generally, disaccharides, such as raffinose and maltose, showed high induction of *SbHRGP3*::*GUS* expression as observed in the sucrose treatment. It appeared that there was no significant difference in the GUS activity induced by non-reducing sugars (raffinose and sucrose) or reducing sugar (maltose). In contrast, monosaccharides showed variable effects on the expression of *SbHRGP3*::*GUS*. All monosaccharides tested in this study showed less inducibility except for glucose in hexoses and xylose in pentoses. Mannitol, an alcohol form of mannose, induced GUS activity comparable to that of the disaccharides described above. Interestingly, *SbHRGP3*::*GUS* induction was observed not only by metabolizable sugars but also by non-metabolizable sugar such as mannose.

Inhibition of photosynthesis and respiration did not affect the wound-inducible expression of *SbHRGP3*::*GUS*

To test whether sucrose serves as an energy source on the wound-induction of *SbHRGP3*, the effect of photosynthesis, from which plants utilize energy for their metabolism, was examined by treatment with DCMU, a specific inhibitor of the light reactions in photosynthesis (Hsu et al. 1986). Wounded leaves were floated on a medium supplemented with sucrose and 20 *μM* DCMU and incubated in the presence or absence of light.

GUS activity was detected in wounded leaves that were floated on a medium supplemented with sucrose in the light (Fig. 3). GUS activity was also observed in the dark, suggesting that the induction of *SbHRGP3* expression was independent of light. Furthermore, DCMU treatment did not affect the wound induction of *SbHRGP3*::*GUS* under light conditions. Dark conditions and inhibition of photosynthesis did not change the inducibility of *SbHRGP3*::*GUS* by sucrose, indicating that the induced expression of *SbHRGP3* is independent of the external energy source.

The role of sucrose on the expression of *SbHRGP3* was also investigated by inhibiting cellular respiration (Kane and Buckley 1977). We used iodoacetate, malonate, and azide to inhibit glycolysis, the Krebs cycle, and oxidative phosphorylation, respectively. After floating the leaf slices on media supplemented with the inhibitors, we measured GUS activity. The result showed that the inhibitors used in this study did not affect enhanced GUS activity, suggesting that sucrose did not act as an internal energy source (Fig. 4). Although azide and iodoacetate treatment rather caused a slight increase in GUS activity, compared with the case without inhibitors, it is not clear that inhibition of glycolysis and oxidative phosphorylation negatively regulates *SbHRGP3* expression from this experiment.

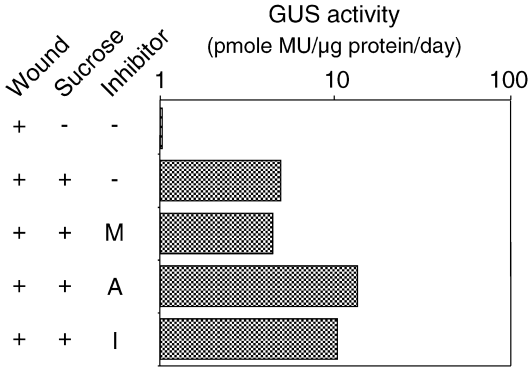

Fig. 4 Effects of various inhibitors blocking cellular energy metabolism on *SbHRGP3*::*GUS* expression. Wounded leaves of transgenic plants were floated on a medium supplemented with sucrose and various inhibitors for 24 h. Thirty micrograms of protein were extracted from each sample and assayed for GUS activity. Wounded leaves floated on a medium supplemented without sucrose were used as a negative control. The result was obtained from a single experiment. *A* Azide, *I* iodoacetate, *M* malonate

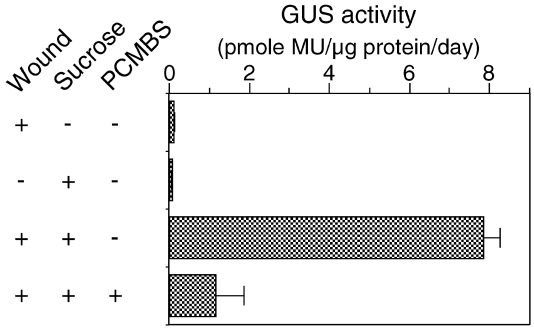

Fig. 5 Effects of PCMBS, an inhibitor of apoplastic sucrose transport, on *SbHRGP3*::*GUS* expression in transgenic plants. Treated (+) and untreated (−) leaf slices of transgenic plants were incubated for 24 h in a medium with or without PCMBS. Thirty micrograms of protein extracted from each sample was assayed for GUS activity. Each mean value was obtained after three independent experiments. *Bars* indicate SDs

PCMBS inhibits the wound-induction of *SbHRGP3*::*GUS*

We have previously shown that *SbHRGP3*::*GUS* was specifically induced in the phloem and cambium in the leaves of transgenic plants by wounding and sucrose treatments (Ahn et al. 1996). The role of sucrose in the expression of *SbHRGP3* in the phloem tissue was examined by blocking active apoplastic transport in transgenic plants. We used PCMBS, a well-known inhibitor of active apoplastic sucrose transport toward phloem (Bush 1993).

The result showed that blocking active apoplastic sucrose transport toward phloem significantly inhibited the expression of *SbHRGP3*::*GUS* (Fig. 5). Induction of *SbHRGP3*::*GUS* decreased by 82% as compared with the control. Therefore, it is likely that the transport of external sucrose molecules toward phloem is a prerequisite for the expression of *SbHRGP3*::*GUS*. However, PCMBS did not completely inhibit *SbHRGP3*::*GUS* induction, suggesting that a small amount of sucrose transported symplastically to the phloem triggered *SbHRGP3*::*GUS* expression or that there may be another minor pathway to induce *SbHRGP3* expression.

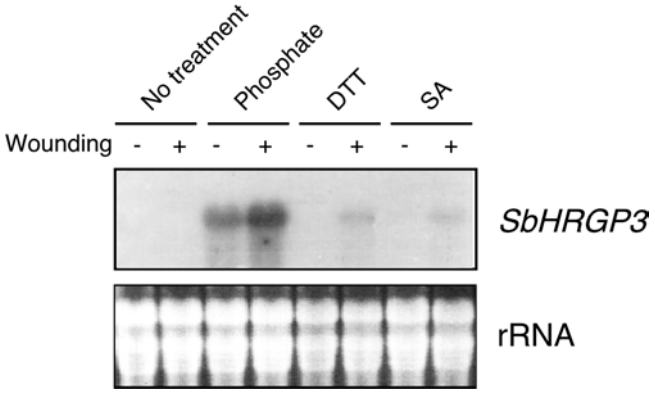

Fig. 6 Effects of external stimuli related to pathogen responses on *SbHRGP3* expression in soybean. Intact and wounded leaves of soybean were treated with the reagents related to pathogen responses including 1 m*M* phosphate, 1 m*M* salicylic acid (*SA*), and 10 m*M* DTT. Ten micrograms of total RNA was analysed by a Northern hybridization analysis. Unwounded leaves and wounded leaves without any treatments were used as negative controls. *Bottom panel* indicates an RNA gel to show an equal amount of total RNA loading

Other external stimuli related to pathogen responses also induce *SbHRGP3* expression in soybean

Because *SbHRGP3* expression was wound-inducible, the effects of other external stimuli related to pathogen responses were examined in soybean. We tested the effects of DTT, salicylic acid, and phosphate on the wound-inducible expression of *SbHRGP3* in soybean leaves.

SbHRGP3 mRNA was not detected in the intact and wounded leaves as we previously reported (Fig. 6). However, DTT, salicylic acid, and phosphate treatments induced *SbHRGP3* expression in the presence of wounding. Neither DTT nor salicylic acid induced *SbHRGP3* expression without wounding. These results were similar to those obtained from wounding and sucrose treatments (Fig. 2). Interestingly, the induced *SbHRGP3* mRNA accumulation was detected in phosphate treatment alone, indicating phosphate treatment bypassed the requirement for sucrose in the wound-inducible expression of *SbHRGP3*. This suggests that phosphate plays an important role in *SbHRGP3* expression in soybean leaves. We also investigated the effect of different phosphate concentrations on *SbHRGP3*::*GUS* in transgenic plants: 100 μM phosphate caused similar levels of induction but >10 m*M* or <10 μM phosphate had no effect on *SbHRGP3*::*GUS* induction in transgenic plants (data not shown).

Discussion

Sugars have long been considered energy sources, which provide ATP through glycolysis pathways and cellular respiration and eventually activate cell metabolism. However, it has recently become apparent that sugars are also a physiological signal affecting the expression of plant genes (Hanson et al. 2001; Jang et al. 1997; Smeekens 1998). We previously found that *SbHRGP3*, a soybean extensin gene whose expression is correlated with root development, requires sucrose for its wound-inducible expression (Ahn et al. 1996, 1998). In this study, we investigated the role of sugars on *SbHRGP3* expression to elucidate whether sugars act as specific regulatory signals or whether they provide energy and affect *SbHRGP3* expression.

The role of sugar on the wound-inducible expression of *SbHRGP3*

Although almost all extensin genes have been reported to be induced by wounding (Elliott and Shirsat 1998; Wycoff et al. 1995), it is unclear why the wound induction of *SbHRGP3* requires sucrose. From the data obtained in this study, it is unlikely that sucrose is required simply for providing energy through glycolysis and cellular respiration to the phloem tissues and cambium cells where *SbHRGP3* expression is observed. This is also inferred from the observation that mannose, which is not utilized during glycolysis but still acts as a potent elicitor for the sugar response (Jang and Sheen 1994), could induce *SbHRGP3* expression (Fig. 2). It is also unlikely that *SbHRGP3* expression was induced by osmotic pressure, because the expression was not induced by sucrose treatment alone.

Sugar signal transduction pathways are well characterized in prokaryotes (Aqvist and Mowbray 1995); however, relatively little is known about the molecular and biochemical mechanisms underlying sugar responses in sugar-producing higher plants (Jang et al. 1997). Because higher plants are autotrophic organisms capable of synthesizing and accumulating sugars from photosynthesis, sugars affecting gene expression have been considered energy sources for growth and development, rather than acting as signal transducers. However, it is still controversial whether sugars act as physiological signals repressing or activating plant genes (Loreti et al. 2000) or whether metabolic flux, rather than sugar levels, is important in triggering sugar response (Borisjuk et al. 2003).

In the signal transduction elicited by sugars, hexokinase was the first molecule identified as a sugar sensor in higher plants (Jang et al. 1997). The enzyme catalyzes the ATP-dependent conversion of hexoses to hexose-6-phosphates, leading to the downstream cascade of sugar signal transduction. The authors suggested that the role of hexokinase in sugar sensing and signalling is conserved evolutionarily although there are structural dissimilarities in the hexokinases of eukaryotes. Recently another component, SUT2, which structurally resembles the yeast sugar sensors SNF3 and RGT2, has been identified as a putative sugar sensor in tomato (Barker et al. 2000).

Sugars also have an important role in wound-inducible expression in various species (Leon et al. 2001). For example, sucrose further enhanced wound-inducibility of mannopine synthase (Ni et al. 1996). The authors showed that the enhancement was conferred by a region of A/T-rich DNA sequences in the promoter of the mannopine synthase gene. Furthermore, expression and accumulation of proteinase inhibitor II of potato were enhanced by sucrose and a 23-mer sequence in the 5′ flanking region of the gene was identified that is capable of restoring the sucrose response as well as the wound response (Kim et al. 1991). Interestingly, *SbHRGP3* contains a sequence motif (GCTTGCT) in the 5′ flanking sequence that is presented in the 23-mer sequence of the proteinase inhibitor II. It appears that sucrose, which may mediate the wound signal transduction by binding to a specific receptor(s) in the cell membrane, leads to *SbHRGP3* expression in the phloem tissues and cambium cells by acting as a specific regulatory signal in the wound signal transduction.

To demonstrate that sugars act as a specific signal, we examined *SbHRGP3*::*GUS* expression when an external or internal source of energy metabolism was blocked in transgenic plants. Photosynthesis and energy-releasing pathways were chosen to block external and internal energy sources, respectively. The results showed that wounding and sucrose in combination still induced *SbHRGP3* expression in the dark, indicating its expression is independent of light. We blocked photosynthesis, an external energy source, by treating DCMU, a specific inhibitor of photosystem II of photosynthesis (Hsu et al. 1986). The wound-induction of *SbHRGP3*::*GUS* was not affected by the inhibitor treatments, suggesting that external energy supplied from photosynthesis is not required for *SbHRGP3* expression in transgenic plants.

We also inhibited cellular respiration to preclude the possibility of the use of sucrose as an internal energy source in *SbHRGP3* expression. We used three inhibitors to inhibit glycolysis, the Krebs cycle, and oxidative phosphorylation because sucrose is converted to glucose and fructose by invertase, which provides initial substrates to ATP-producing cellular metabolism (Salisbury and Ross 1992). The results showed that the inhibitor treatments did not affect the wound-inducible expression of *SbHRGP3* in transgenic plants, implying that sucrose is not required as an internal energy source for *SbHRGP3* expression (Fig. 4).

Sucrose as a signalling molecule was further investigated by treating PCMBS, an inhibitor of active apoplastic transport of sucrose toward phloem. Leguminous plants including soybean contain a few plasmodesmata leading into the companion cells and the major transport compound in phloem exudate is sucrose (Turgeon and Medville 1998), indicating that apoplastic transport of sucrose is a major way to translocate photosynthetates in

soybean. If sucrose acts as a signal to trigger signal transduction in the tissues, sucrose should be translocated into the phloem tissues to induce *SbHRGP3* expression. Our results showed that, when the apoplastic transport of sucrose was blocked, expression of *SbHRGP3* was inhibited in transgenic plants. This result implies that *SbHRGP3* expression requires the translocated sucrose, suggesting that the translocated sucrose triggers the wound-inducible expression of *SbHRGP3*. Taken together, our data suggest that sugars including sucrose act as a specific regulatory signal on *SbHRGP3* expression, rather than as a simple provider of energy to the tissues.

However, this argument leads to a question concerning the metabolic inhibitors used in this study because their target-specificities have not been fully elucidated in plants. Although there are a number of inhibitors that work, it is not plausible to conclude that sugars act as a signal from an experiment in which a single inhibitor was used. However, if similar results were obtained from several inhibitors affecting cellular metabolism, it is reasonable to expect that *SbHRGP3* expression is independent of cellular metabolism and sugars are not required to provide energy on the wound-inducible expression of the gene.

Other stimuli related to pathogen responses also induce *SbHRGP3* expression

Expression of extensin genes was generally affected by external factors such as sulfhydryl reagents, reducing agents, chemical elicitors, salicylic acid, and elevated H_2O_2 level (Showalter 1993). We tested some of them in relation to the wound response on *SbHRGP3* expression. We used strong reducing agents that generally induce activation of plant defence genes, especially DTT that can reverse the inhibitory effect of PCMBS on the wound-inducible expression (Narvaez-Vasquez et al. 1994). The results showed that neither salicylic acid nor DTT treatment induced *SbHRGP3* expression although those treatments activate many genes that are wound-inducible (Fig. 6). These results suggest that the expression of *SbHRGP3* in soybean involves a rather unique pathway to transduce the wound signal.

Another interesting feature is that phosphate treatment induced *SbHRGP3* expression in intact soybean leaves without wounding (Fig. 6). The role(s) of phosphate in the wound response is not clear yet; however, phosphate is an important factor in wound response because some of the earliest physiological events after wounding included a change in intracellular phosphate concentrations (Strasser and Matern 1986). Although phosphate affects the wound-inducible expression in various ways, similarities in the expression pattern concerning sucrose enhancement have emerged between a vegetative storage protein (AtVSP) from *Arabidopsis* (Berger et al. 1995) and *SbHRGP3*. Further studies are required to explicate the role of phosphate in the wound response and its relation to the signal transduction pathway leading to the *SbHRGP3* expression.

SbHRGP3 is regulated in both tissue-specific and development-specific manners in soybean (Ahn et al. 1996, 1998), suggesting that more than a single signal transduction pathway is involved in the regulation of the gene. Although *SbHRGP3* expression is wound-inducible, it requires sugars for its wound-inducible expression. Interestingly, inhibition of photosynthesis and cellular respiration did not affect *SbHRGP3*::*GUS* expression although inhibition of active apoplastic transport of sucrose did. Based on the results of this study, it is likely that sugars act as specific regulatory signals in the wound-inducible expression of *SbHRGP3*, rather than as simple providers of energy to the tissues. The requirement for sucrose and the dependency on sulfhydryl reagents, salicylic acid, and phosphate will also provide clues toward understanding the mechanisms of regulation of *SbHRGP3* expression. Identification and further characterization of the components interacting with *SbHRGP3* will shed light on the complicated wound signal transduction networks of *SbHRGP3*.

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